Insights into electroreceptor development and evolution from molecular comparisons with hair cells

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Synopsis

The vertebrate lateral line system comprises a mechanosensory division, with neuromasts containing hair cells that detect local water movement ("distant touch"); and an electrosensory division, with electrosensory organs that detect the weak, low-frequency electric fields surrounding other animals in water (primarily used for hunting). The entire lateral line system was lost in the amniote lineage with the transition to fully terrestrial life; the electrosensory division was lost independently in several lineages, including the ancestors of frogs and of teleost fishes. (Electroreception with different characteristics subsequently evolved independently within two teleost lineages.) Recent gene expression studies in a non-teleost actinopterygian fish suggest that electroreceptor ribbon synapses employ the same transmission mechanisms as hair cell ribbon synapses, and show that developing electrosensory organs express transcription factors essential for hair cell development, including Atoh1 and Pou4f3. Previous hypotheses for electroreceptor evolution suggest either that electroreceptors and hair cells evolved independently in the vertebrate ancestor from a common ciliated secondary cell, or that electroreceptors evolved from hair cells. The close developmental and putative physiological similarities implied by the gene expression data support the latter hypothesis, i.e., that electroreceptors evolved in the vertebrate ancestor as a "sister cell-type" to lateral line hair cells.

The mechanosensory division of the lateral line system

The sensory lateral line system (Coombs et al., 1989; Bullock et al., 2005; Coombs et al., 2014) only functions in water, and was lost in the lineage leading to amniotes; it is found in all fishes and aquatic-stage amphibians (except for myxinid hagfishes and some direct-developing amphibians lacking an aquatic larval stage, in which it was lost secondarily; Bullock et al., 1983; Braun and Northcutt, 1997; Schlosser, 2002b). The mechanosensory division comprises lines of "neuromasts" along the flank (hence "lateral line") and around the head, often buried in canals that open to the water via pores (Webb, 2014). The sensory cells of neuromasts are hair cells, which morphologically resemble mammalian type 2 vestibular hair cells; like all hair cells, they are stimulated by deflection of the apical "hair bundle", i.e., a

staircase array of actin-rich microvilli ("stereocilia"), in the direction of the tallest stereocilia and adjacent primary cilium ("kinocilium") (Chagnaud and Coombs, 2014; Nicolson, 2017). This action triggers cation entry through a mechanically gated channel, whose identity is still controversial (Corey and Holt, 2016; Fettiplace, 2016; Wu and Müller, 2016) (see also Wu et al., 2017), depolarising the hair cell. This opens $Ca_V1.3$ channels (L-type voltage-gated Ca^{2+} channels) in the basolateral membrane, clustered beneath "pre-synaptic ribbons": proteinaceous structures that tether many glutamate-filled synaptic vesicles, enabling rapid, sustained neurotransmitter release at these "ribbon synapses" (Safieddine et al., 2012; Nicolson, 2015; Wichmann and Moser, 2015) with the nerve terminals of afferent lateral line neurons, whose cell bodies are collected in lateral line ganglia. Mechanosensory lateral line afferents project via the anterior or posterior lateral line nerves (depending on the position of the neuromasts) to the medial octavolateral nucleus in the hindbrain (Wullimann and Grothe, 2014). The mechanosensory lateral line responds to water movement relative to the body's surface, hence mediates a sense of "distant touch" (Dijkgraaf, 1963; Montgomery et al., 2014).

It has long been known from descriptive and fate-mapping studies in amphibian and, more recently, zebrafish embryos, that neuromasts and their afferent neurons develop from lateral line placodes - paired patches of thickened neurogenic ectoderm on the head (see e.g. Wright, 1951; Schlosser, 2002a; Piotrowski and Baker, 2014). Over 120 years ago, for example, Julia Platt memorably described watching the posterior lateral line placode of the urodele amphibian *Necturus* "plough its way through the skin, leaving a row of sense-organs in the wake" (Platt, 1896). Three developmentally and evolutionarily independent sets of paired cranial neurogenic placodes form hair cell-containing organs and their afferent neurons: the otic placode forms the inner ear (Alsina and Whitfield, 2017; Gálvez et al., 2017), the paired spiracular/paratympanic organ placode forms the anamniote spiracular organ and its amniote homologue (where present), the paratympanic organ (O'Neill et al., 2012), and the lateral line placodes form lateral line organs. All these placodes develop as thickenings within the "posterior placodal area" adjacent to the hindbrain (Grocott et al., 2012; Saint-Jeannet and Moody, 2014; Schlosser, 2014). Phylogenetic analysis suggests that the ancestor of all jawed vertebrates had three

lateral line placodes rostral to the otic placode, and three caudal to the otic placode (Northcutt, 1997). After forming neuroblasts, which delaminate and coalesce with neural crest-derived glia to form lateral line ganglia (which may in turn fuse with other nearby ganglia), lateral line placodes either elongate to form sensory ridges over the head, or migrate as a cell collective along the trunk, tracked by afferent axons and associated Schwann cells (Wright, 1951; Schlosser, 2002a; Piotrowski and Baker, 2014). The lateral line system of the teleost zebrafish is a widely used model for hair cell development and also regeneration, for example after treatment with ototoxic drugs such as aminoglycoside antibiotics (Thomas et al., 2015; Kniss et al., 2016; McGraw et al., 2017).

The electrosensory division of the lateral line system

Electroreception, i.e., the ability to detect weak electric fields in water, was only discovered in the mid-twentieth century, in conjunction with the finding that "weakly electric" teleost fishes generate electric fields (Lissmann, 1951; Lissmann and Machin, 1958; Lissmann, 1958) by discharging "electric organs" composed of modified muscle cells (Markham, 2013; Gallant et al., 2014). Weakly electric fishes have diverse "tuberous" electrosensory organs stimulated by the high-frequency electric fields generated by electric organ discharges, and morphologically distinct "ampullary" organs stimulated by the low-frequency electric fields around other animals (Bodznick and Montgomery, 2005; Jørgensen, 2005). (Tiny direct-current standing electric fields develop around animals in water owing to the leakage of ions across mucous membranes, with a low-frequency component imparted by, for example, rhythmic limb or ventilation movements; Wilkens and Hofmann, 2005; Bedore and Kajiura, 2013.) Teleost electroreceptors respond to anodal (exterior-positive) stimuli and are inhibited by cathodal stimuli, and are innervated (depending on their position) by either anterior or posterior lateral line nerves projecting to distinct "electrosensory lateral line lobes" in the medulla (Bullock et al., 1983; New, 1997; Alves-Gomes, 2001; Baker et al., 2013).

Following the discovery of electroreception in weakly electric teleosts, electroreception with different characteristics of physiology and innervation was discovered in representatives of all major

vertebrate groups: stimulation by weak, low-frequency cathodal (exterior-negative) electric fields, inhibition by weak anodal (exterior-positive) stimuli, and innervation by lateral line afferents projecting to the dorsal octavolateral nucleus in the medulla via the dorsal root of the anterior lateral line nerve (Bullock et al., 1983; New, 1997; Baker et al., 2013). Electroreception with all these characteristics is present in lampreys within the agnathan (jawless) fishes (which molecular evidence overwhelmingly supports as a monophyletic clade, the cyclostomes; Kuraku, 2008; Heimberg et al., 2010; Shimeld and Donoghue, 2012; Fig. 1), and across all three gnathostome (jawed) vertebrate groups: in all chondrichthyans (cartilaginous fishes), and in lineages within both groups of osteichthyans (bony fishes), i.e., in coelacanths, dipnoans (lungfishes) and both urodele and caecilian amphibians within the sarcopterygians (lobe-finned bony fishes and tetrapods), and in polypterids (bichirs, reedfishes) and chondrosteans (sturgeons, paddlefishes) within the actinopterygians (ray-finned bony fishes) (Bullock et al., 1983; New, 1997; Baker et al., 2013) (Fig. 1).

The most parsimonious hypothesis to explain this distribution is that electroreception was present in the ancestor of all living vertebrates, but lost independently in the agnathan lineage leading to hagfishes, in the sarcopterygian lineage leading to anuran amphibians, and in the actinopterygian lineage leading to the neopterygian fishes, i.e., holosteans (gars, bowfin) and teleosts (Bullock et al., 1983; New, 1997; Baker et al., 2013) (Fig. 1). Teleost electroreception is clearly different (see above), and only two related groups within each of two distinct lineages are electroreceptive, with one of the two groups in each lineage also having evolved electric organs and tuberous electroreceptors, likely representing multiple independent evolutionary events, probably via the diversification of neuromast hair cells (Bullock et al., 1983; New, 1997; Baker et al., 2013). (Within the amniotes, monotreme mammals and dolphins independently evolved trigeminal nerve-mediated electroreception, which will not be considered further here; for more information on this system, see Czech-Damal et al., 2013.)

Like hair cells, electroreceptors in all vertebrates have basolateral ribbon synapses with the terminals of their afferent neurons (Jørgensen, 2005). (Unlike hair cells, electroreceptors lack efferent innervation; Bodznick and Montgomery, 2005.) Electroreceptors in non-teleost fishes and amphibians

resemble immature hair cells (Northcutt et al., 1994) in having an apical primary cilium and variable numbers of microvilli (from none to hundreds, depending on the species) (Jørgensen, 2005), which are not organised into the stepped "hair bundle" that characterises mature hair cells (Lu and Sipe, 2016). In contrast, electroreceptors in the jawless lampreys and all teleosts bar one species have apical microvilli but no primary cilium (Jørgensen, 2005). (Lamprey electroreceptor development has not been characterised, however, so it is possible that a primary cilium forms but is lost during maturation, as in mammalian cochlear hair cells; Lu and Sipe, 2016.) (Nothing is known about the function of microvilli in electroreceptors, or why they should be present in some taxa but not others.) Lamprey electroreceptors are collected into superficial "end-bud" electrosensory organs, while in non-teleost gnathostomes, electroreceptors interspersed with supporting cells comprise the sensory epithelium in "ampullary organs": flask-shaped expansions at the base of a duct filled with conductive jelly (see e.g. Josberger et al., 2016), which opens to the surface via a pore (Jørgensen, 2005).

Over the last quarter-century, fate-mapping studies across the three major gnathostome groups have revealed that individual lateral line placodes form ampullary organs, as well as neuromasts and lateral line afferent neurons (Baker et al., 2013). These involved ablation and grafting studies in embryos of a sarcopterygian (a urodele amphibian, the axolotl *Ambystoma mexicanum*) (Northcutt et al., 1995), and vital dye (DiI)-labelling experiments in an actinopterygian (a chondrostean, the Mississippi paddlefish *Polyodon spathula*) (Modrell et al., 2011) and a chondrichthyan (the little skate, *Leucoraja erinacea*) (Gillis et al., 2012). The placodes elongate to form sensory ridges, which ultimately fragment, with a line of neuromasts forming first, along the centre of the ridge, and ampullary organs forming later, from both flanks, such that fields of ampullary organs flank the neuromast line (Piotrowski and Baker, 2014). Furthermore, lateral line (and otic) placodes in all three species, as originally shown in another chondrichthyan, the shark *Scyliorhinus canicula*, express the transcriptional coactivator and phosphatase gene *Eya4*, and maintain *Eya4* expression in both electroreceptors and hair cells (O'Neill et al., 2007; Modrell et al., 2011; Gillis et al., 2012; Modrell and Baker, 2012).

Their shared embryonic origin in all three gnathostome groups, plus the shared characteristics of physiology and innervation as described earlier, together provide strong evidence for the homology of ampullary organs and electroreceptors across gnathostomes. Much less is known about agnathan electroreceptor development. Although sensory neurons derived from lamprey cranial placodes, including lateral line neurons, have been fate-mapped using focal DiI-labelling (Modrell et al., 2014), neither electroreceptors nor neuromasts have been identified at embryonic stages (though both are functional in ammocoete larvae; Ronan, 1988; Gelman et al., 2007). Thus, the embryonic origin of lamprey electroreceptors remains undetermined (as does that of teleost electroreceptors, though descriptive studies in a catfish suggest that ampullary organs form on the flanks of thin sensory ridges formed by elongating lateral line placodes, as in non-teleosts; see Northcutt, 2003; Baker et al., 2013). Nevertheless, the conserved physiology and innervation of lamprey and non-teleost gnathostome electroreceptors strongly supports their homology, i.e., that the electrosensory division of the lateral line system was inherited, like the mechanosensory division, from the common ancestor of all living vertebrates.

Molecular analysis suggests that electroreceptor synaptic transmission mechanisms are conserved with hair cells

As noted above, hair cells and electroreceptors are characterised by basolateral ribbon synapses with afferent nerve terminals, with presynaptic ribbons of varying morphologies (Jørgensen, 2005). The presynaptic ribbon is mainly composed of the only ribbon-specific protein known, Ribeye: this is produced from an alternative start site for the NAD(H)-sensitive transcriptional corepressor gene *Ctbp2*, resulting in an N-terminal A-domain found only in Ribeye (Schmitz et al., 2000; Stankiewicz et al., 2014). Vertebrate retinal and pineal photoreceptors, and retinal bipolar neurons, also have ribbon synapses (Zanazzi and Matthews, 2009; Matthews and Fuchs, 2010; Mercer and Thoreson, 2011; Safieddine et al., 2012). Presynaptic ribbons also contain conventional presynaptic cytomatrix proteins like Piccolo and Bassoon: in both photoreceptors and hair cells, the latter is found at the base of the ribbon, and is

required to anchor the ribbon to the plasma membrane (Zanazzi and Matthews, 2009; Matthews and Fuchs, 2010; Mercer and Thoreson, 2011; Safieddine et al., 2012). In the absence of Ribeye, the ribbon is lost and neurotransmitter release is reduced, but not abolished, at both retinal and hair cell synapses (Lv et al., 2016; Maxeiner et al., 2016; Becker et al., 2018; Jean et al., 2018). Thus, presynaptic ribbons may simply represent "an extreme example of the more general need for presynaptic machinery to facilitate the supply of release-ready vesicles to the active zone" (Matthews and Fuchs, 2010).

As described below, hair cell ribbon synapse physiology differs from other ribbon synapse physiology in multiple respects (Zanazzi and Matthews, 2009; Matthews and Fuchs, 2010; Mercer and Thoreson, 2011; Safieddine et al., 2012). Furthermore, as detailed below, our recent analysis of the expression of ribbon synapse-associated genes in a non-teleost actinopterygian suggests that electroreceptor ribbon synapses share the "unique" physiology of hair cell ribbon synapses (Modrell et al., 2017). These transcripts were identified in a set of just under 500 genes identified by differential RNA-seq as being enriched at least 1.85-fold in pooled operculum (gill-flap: covered in ampullary organs, plus some neuromasts) versus fin tissue (similar in tissue composition but without any lateral line organs) at late larval stages (the onset of independent feeding) in a chondrostean, the paddlefish *Polyodon spathula* (Modrell et al., 2017).

In retinal photoreceptors, depolarisation elicits synaptic vesicle exocytosis by opening $Ca_v1.4$ channels (L-type voltage-gated Ca^{2+} channels) clustered near the presynaptic ribbon (Safieddine et al., 2012; Joiner and Lee, 2015). In hair cells, this role is played by $Ca_v1.3$ channels (whose pore-forming alpha subunit is encoded by *Cacna1d*) (Safieddine et al., 2012; Joiner and Lee, 2015; Nicolson, 2015), with the auxiliary subunit $Ca_v\beta2$ (encoded by *Cacnb2*) regulating their number and function (Neef et al., 2009). The only Ca^{2+} channel subunit genes present in the paddlefish lateral line-enriched gene-set were *Cacna1d* and *Cacnb2* (Modrell et al., 2017). *In situ* hybridisation (at the same late-larval stage used for RNA-seq) revealed that, in addition to expression in neuromasts, as expected (Nicolson, 2015; 2017), both *Cacna1d* and *Cacnb2* were expressed in ampullary organs (Fig. 2A-C; Modrell et al., 2017). This suggests that synaptic vesicle exocytosis is triggered in electroreceptors, as in hair cells, by $Ca_v1.3$

channels (using the same $Ca_v\beta2$ subunit as in hair cells) rather than the $Ca_v1.4$ channels used at retinal photoreceptor ribbon synapses (Safieddine et al., 2012; Joiner and Lee, 2015). [Intriguingly, $Ca_v1.3$ was also recently identified as the apically located voltage-sensing Ca^{2+} channel responsible for electroreceptor function in a chondrichthyan, the little skate *Leucoraja erinacea* (Bellono et al., 2017), raising the possibility that $Ca_v1.3$ could also be the voltage-sensor for paddlefish electroreceptors (also see Modrell et al., 2017).]

In all ribbon synapses except hair cells, and at excitatory glutamatergic synapses in the central nervous system, the vesicular glutamate transporters Vglut1(encoded by *Slc17a7*) and/or Vglut2 (encoded by *Slc17a6*) are required to fill synaptic vesicles (Zanazzi and Matthews, 2009; Pangršič et al., 2012). In contrast, Vglut3 (encoded by *Slc17a8*) performs this function at hair cell ribbon synapses (Obholzer et al., 2008; Ruel et al., 2008; Seal et al., 2008). In the paddlefish lateral line-enriched gene-set, the only *Slc17a* gene family member present was *Slc17a8*, encoding Vglut3 (Modrell et al., 2017): this proved to be expressed in ampullary organs as well as neuromasts (Fig. 2D; Modrell et al., 2017). This finding suggests that in electroreceptors, as in hair cells but not in other ribbon synapse-containing cells, Vglut3 loads synaptic vesicles with neurotransmitter.

Furthermore, synaptic vesicle exocytosis is mediated at retinal ribbon synapses, as at other synapses, by neuronal SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors), whereas at hair cell ribbon synapses, SNAREs are replaced by a calcium-sensitive type II ferlin, otoferlin (Hams et al., 2017; Michalski et al., 2017) (also see Pangršič et al., 2012). The gene encoding otoferlin, *Otof*, was present in the paddlefish lateral line-enriched gene-set and expressed in ampullary organs as well as neuromasts (Fig. 2E; Modrell et al., 2017), suggesting that otoferlin mediates synaptic vesicle exocytosis at the ribbon synapses of both electroreceptors and hair cells.

Finally, although not unique to hair cell ribbon synapses, the paddlefish study identified the expression in ampullary organs (as well as neuromasts) of *Rims2*, encoding Rab3-interacting molecules 2 α and β, which are necessary to recruit $Ca_v1.3$ channels to the active zone membrane under the presynaptic ribbon of cochlear inner hair cells (Jung et al., 2015) (Fig. 2F; Modrell et al., 2017) and

Ribeye, identified by *in situ* hybridisation using a riboprobe against the N-terminal A-domain sequence of paddlefish *Ctbp2* (Fig. 2G; Modrell et al., 2017).

Taken together, these gene expression studies, albeit descriptive, suggest that the synaptic transmission mechanisms at the electroreceptor ribbon synapse are conserved with those of hair cells.

Developing ampullary organs express transcription factors essential for hair cell development, including *Atoh1* and *Pou4f3* (*Brn3c*)

The paddlefish lateral line-enriched gene-set included several transcription factor genes important for mammalian hair cell development (Modrell et al., 2017). Previous candidate gene approaches had already identified the expression of such genes in developing ampullary organs, as well as neuromasts, including the “pan-placodal” homeodomain transcription factor gene *Six1* and its transcription coactivator gene *Eya1* (Fig. 3A,B; Modrell et al., 2011). During cochlear hair cell development, Six1 and Eya1 interact physically with the high mobility group domain transcription factor Sox2 to form a complex that is capable of inducing expression of the basic helix-loop-helix (bHLH) transcription factor gene *Atoh1* in cochlear explants (Ahmed et al., 2012; Zhang et al., 2017). Atoh1 is necessary for hair cell formation (Bermingham et al., 1999) and is sufficient to promote hair cell differentiation in the context of the inner ear (Cai and Groves, 2015; Jahan et al., 2015; Costa et al., 2017). Both *Sox2* and *Atoh1* were also present in the paddlefish lateral line-enriched gene-set and proved to be expressed in developing ampullary organs, as well as neuromasts (Fig. 3C,D; Modrell et al., 2017). (*Atoh1* expression in developing ampullary organs was also reported briefly in a paper on paddlefish cerebellum development; Butts et al., 2014) Similarly, the POU domain transcription factor gene *Pou4f3* (*Brn3c*), which is essential for hair cell formation (Erkman et al., 1996; Xiang et al., 1997), and is induced in the cochlea by Six1 and Eya1, independently of Atoh1 (Ahmed et al., 2012), was in the paddlefish lateral line-enriched gene-set and expressed in developing ampullary organs (Fig. 3E; Modrell et al., 2017).

The expression of all these transcription factor genes in developing ampullary organs suggests that they are likely to be important for electroreceptor differentiation, not just hair cell differentiation.

Indeed, Atoh1 is required for the differentiation of a variety of other cell types, including mechanosensory Merkel cells and proprioceptive neurons, plus cerebellar granule neurons and intestinal secretory cells: its precise role in directing hair cell development is surprisingly little understood (Cai and Groves, 2015; Jahan et al., 2015; Costa et al., 2017). Another bHLH transcription factor gene present in the paddlefish lateral line-enriched gene-set, *Neurod4*, may be important specifically for electroreceptor development, as its expression was restricted to developing ampullary organs, and never seen in neuromasts (Fig. 3F-H[2]; Modrell et al., 2017).

Although all these studies are descriptive, testing the function of these transcription factors and other genes during electroreceptor versus hair cell development is a very real prospect, given the efficiency of CRISPR/Cas9 genome-editing tools injected into fertilised eggs of urodele amphibians (so far, the axolotl and a newt, *Pleurodeles waltl*; see e.g. Flowers et al., 2014; Elewa et al., 2017). It may also be possible to target lateral line placodes directly as Cas9 protein/guide RNA complexes can also be electroporated into target tissue, resulting in very efficient genome editing (Fei et al., 2016). Such approaches could, in principle, be applied to any electroreceptive species for which embryos can be obtained frequently and in sufficiently large numbers to enable optimisation of CRISPR/Cas9 for that species.

Implications for electroreceptor evolution in the vertebrate ancestor

As proposed almost 40 years ago, based primarily on morphology (Jørgensen, 1982; 1989), electroreceptors could have evolved in the vertebrate ancestor via the diversification of already-evolved lateral line hair cells, or hair cells and electroreceptors could have been derived independently from a ciliated axon-less sensory cell, which itself probably evolved from an ancestral primary sensory neuron (see also Fritzsch and Straka, 2014). Indeed, putative hair cell homologues have been reported in tunicates (the closest invertebrate chordate relatives of the vertebrates; Fig. 1), as axon-less (secondary) sensory cells with one or more apical cilia and microvilli, afferent glutamatergic synapses, and conservation of some gene expression with hair cells (Burighel et al., 2003; 2011; Rigon et al., 2013;

2018), located in the coronal organ of the oral siphon tentacles and velum in ascidians, and in the circum-oral ring of appendicularians (Burighel et al., 2003; 2011; Rigon et al., 2013; 2018). Physiological evidence suggests that the ascidian coronal organ is mechanosensory (Mackie et al., 2006). It is plausible that the common ancestor of tunicates and vertebrates had scattered secondary mechanosensory cells, which evolved in the tunicate lineage into coronal organ cells in ascidians and circum-oral ring cells in appendicularians, and into hair cells (and electroreceptors) in vertebrates, even though the former (which are associated with oral regions) develop from more rostral ectoderm than vertebrate hair cells, which all develop from placodes forming from the posterior placodal area adjacent to the hindbrain (for a more detailed discussion, see Patthey et al., 2014; Schlosser et al., 2014; Schlosser, 2015).

However, the ribbon synapses that are characteristic of hair cells and electroreceptors (as well as retinal and pineal photoreceptors, and retinal bipolar neurons) seem to be vertebrate-specific: they have not been described in either tunicates (including the putative hair cell homologues; Burighel et al., 2003; Rigon et al., 2018) or the cephalochordates (amphioxus species) (see e.g. Petralia et al., 2015; 2016; 2017). Several independent lines of evidence support the homology of the frontal eye of amphioxus to the vertebrate retina (Lacalli, 1996; Vopalensky et al., 2012; Suzuki et al., 2015; Pergner and Kozmik, 2017), while the amphioxus lamellar body (located in the roof of the larval cerebral vesicle) is thought to be homologous to the vertebrate pineal organ (Eakin and Westfall, 1962; Ruiz and Anadón, 1991; Pergner and Kozmik, 2017). Thus, ribbon-less cellular homologues of retinal and, most likely, pineal photoreceptors were already present in separate locations within the central nervous system of the ancestral chordate. Taken together with the physiological differences between ribbon synapses in retinal versus hair cells (Zanazzi and Matthews, 2009; Matthews and Fuchs, 2010; Mercer and Thoreson, 2011; Safieddine et al., 2012), this suggests that ribbon synapse-containing cells could not all have evolved from a common ancestral cell type. Instead, the recruitment of Ribeye to the presynaptic active zone machinery, and thus the formation of presynaptic ribbons, must represent independent evolutionary events during the evolution of vertebrate retinal cells, pineal cells and hair cells. In all these sensory cell

types, increasing the number of synaptic vesicles available for ready release - the primary function of the ribbon (Lv et al., 2016; Maxeiner et al., 2016; Becker et al., 2018; Jean et al., 2018) - would have been a selective advantage.

The gene expression data from paddlefish suggest that ribbon synapses in electroreceptors use hair cell-specific transmission mechanisms (i.e., involving $Ca_v1.3$, Vglut3 and otoferlin; Fig. 2; Modrell et al., 2017). Similarly, the fact that so many transcription factor genes essential for hair cell differentiation, including *Atoh1* and *Pou4f3*, are also expressed in developing ampullary organs (Fig. 3; Modrell et al., 2011; 2017) suggests a very close developmental and evolutionary relationship with hair cells. Taking all these lines of evidence together, and with the caveat that nothing is yet known at the molecular level about electroreceptors in lampreys, it seems less likely that electroreceptors and hair cells evolved independently from a ciliated secondary sensory cell in the vertebrate ancestor, as this would also require electroreceptors to have recruited Ribeye to their presynaptic active zones, and formed presynaptic ribbons, independently of hair cells. Instead, given all the characteristics shared with hair cells, it seems more parsimonious to suggest that lateral line electroreceptors evolved in the vertebrate ancestor as a sister cell-type to lateral line hair cells (Arendt et al., 2016), i.e., via the diversification of lateral line hair cells.

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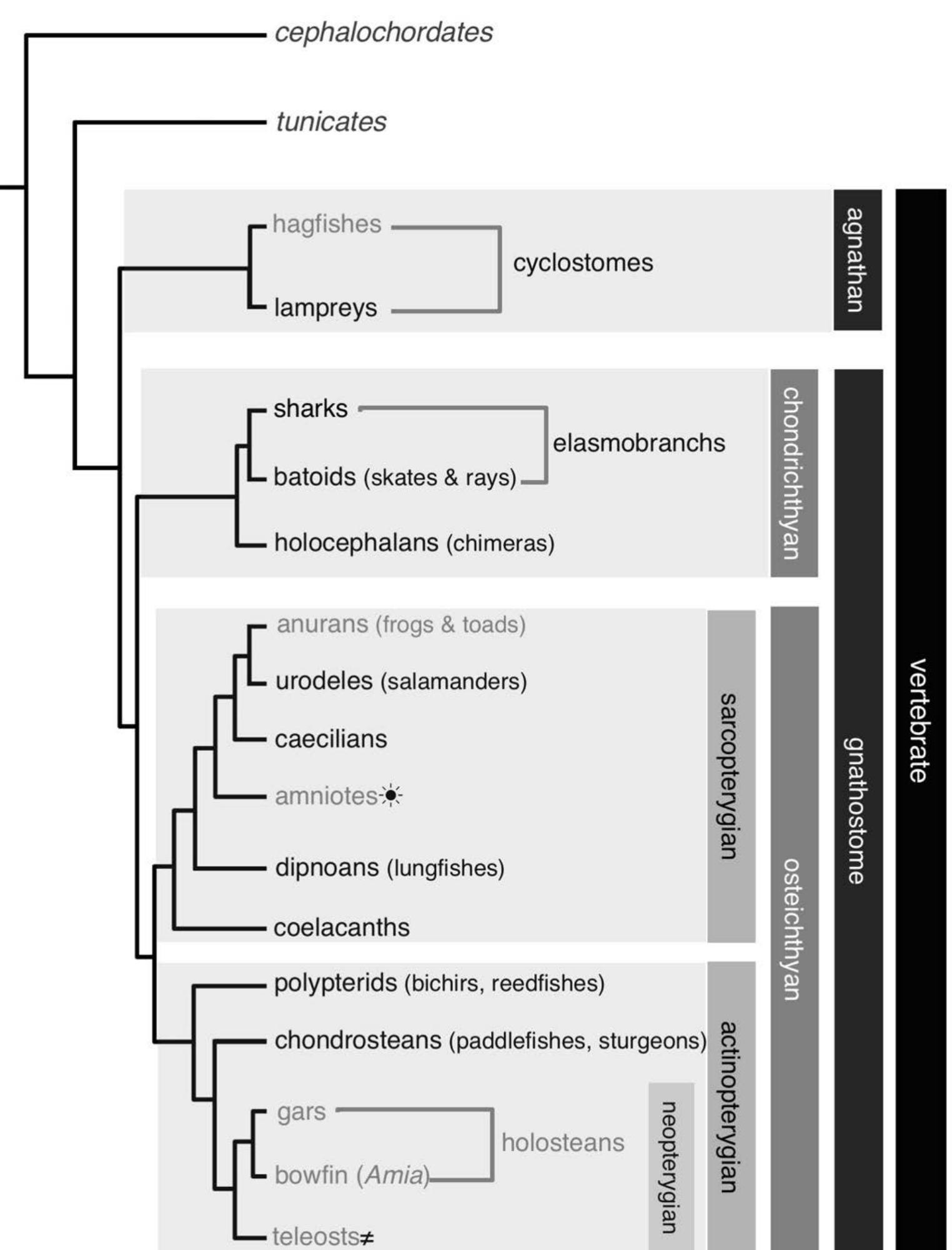
Figure Legends

Fig. 1 Phylogeny showing the distribution of lateral line sensory divisions among living vertebrates, with the invertebrate chordates shown for reference. Black font indicates possession of both the mechanosensory division and the electrosensory division (the latter responding to low-frequency, cathodal stimuli, with the dorsal octavolateral nucleus as the hindbrain target of lateral line afferents projecting via the dorsal root of the anterior lateral line nerve). Grey font indicates possession of the mechanosensory division only, except for the amniotes (☀), which lost the whole lateral line system during the transition to fully terrestrial life, and actinopterygian teleost fishes (≠), in which a few groups independently evolved lateral line electroreception responding to anodal stimuli, with an electrosensory lateral line lobe as the hindbrain target of lateral line afferents projecting via both anterior and posterior lateral line nerves.

Fig. 2 Genes important for transmission at the hair cell ribbon synapse are expressed in electrosensory organs in the paddlefish. (**A**) Schematic showing the distribution of neuromasts in canals, flanked by large fields of electrosensory ampullary organs, on the head of a late-larval paddlefish (at the onset of independent feeding). The caudal box outlines the region shown at higher power in panels C,D; the rostral box outlines the region shown at higher power in panels E-G. (**B-G**) *In situ* hybridisation (shown in skin-mounts in panels C-G: white dotted lines indicating the approximate position of lateral line canals, within which neuromasts are buried) reveals expression of hair cell ribbon synapse-associated genes in ampullary organs, as well as neuromasts, for (B) *Cacna1d*, encoding the pore-forming subunit of $Ca_v1.3$; (C) *Cacnb2*, encoding the $Ca_v\beta2$ auxiliary subunit for $Ca_v1.3$ in hair cells (expression is very weak in neuromasts: compare with panel D for neuromast distribution); (D) *Scl17a8*, encoding Vglut3 (asterisk indicates damage to the skin-mount); (E) *Otof*, encoding otoferlin; (F) *Rims2*, encoding Rab3-interacting molecules 2 α and β, which recruit $Ca_v1.3$ channels to the membrane under the presynaptic ribbon in hair cells; (G) the N-terminal A-domain sequence of *Ctbp2*, encoding Ribeye. Scale-bars: 200 μm in B; 100 μm in C (panels D-G are shown at the same magnification as panel C). Abbreviations: ao, ampullary organs; e, eye; io, infraorbital neuromast line; nm, neuromasts; ol, otic neuromast line; so, supraorbital neuromast line. (A colour version of this figure is available online.)

Fig. 3 Transcription factor genes important for hair cell development are expressed in developing electrosensory organs in the paddlefish. (**A-E**) *In situ* hybridisation in paddlefish embryos reveals expression in both developing neuromasts and ampullary organs of (A) *Six1* and (B) *Eya1* (expression of both is also seen in the posterior lateral line primordium migrating along the trunk); (C) *Sox2*, (D) *Atoh1* (the inset shows neuromast lines at an earlier stage of development) and (E) *Pou4f3* (*Brn3c*). (**F-H^2**) The bHLH transcription factor gene *Neurod4* is expressed in ampullary organs but not neuromasts. The dotted white lines in panel G show the approximate position of the neuromast lines. H-H^2 show a transverse section through the head (near the eye). Panel H shows *Neurod4* expression in an ampullary organ. Panel H^1 shows immunostaining for a calcium-buffering protein (an oncomodulin-related beta-parvalbumin; see Modrell et al., 2017) expressed in both neuromast hair cells and electroreceptors, which shows a *Neurod4*-negative neuromast dorsal to a *Neurod4*-positive ampullary organ. Panel H^2 shows DAPI, which stains all nuclei. Scale-bars: 500 μm in A (panels B-E shown at the same magnification as panel A); 500 μm in F; 100 μm in G; 50 μm in H. Abbreviations: ao, ampullary organ; e, eye; nm, neuromasts; pll, posterior lateral line primordium. (A colour version of this figure is available online.)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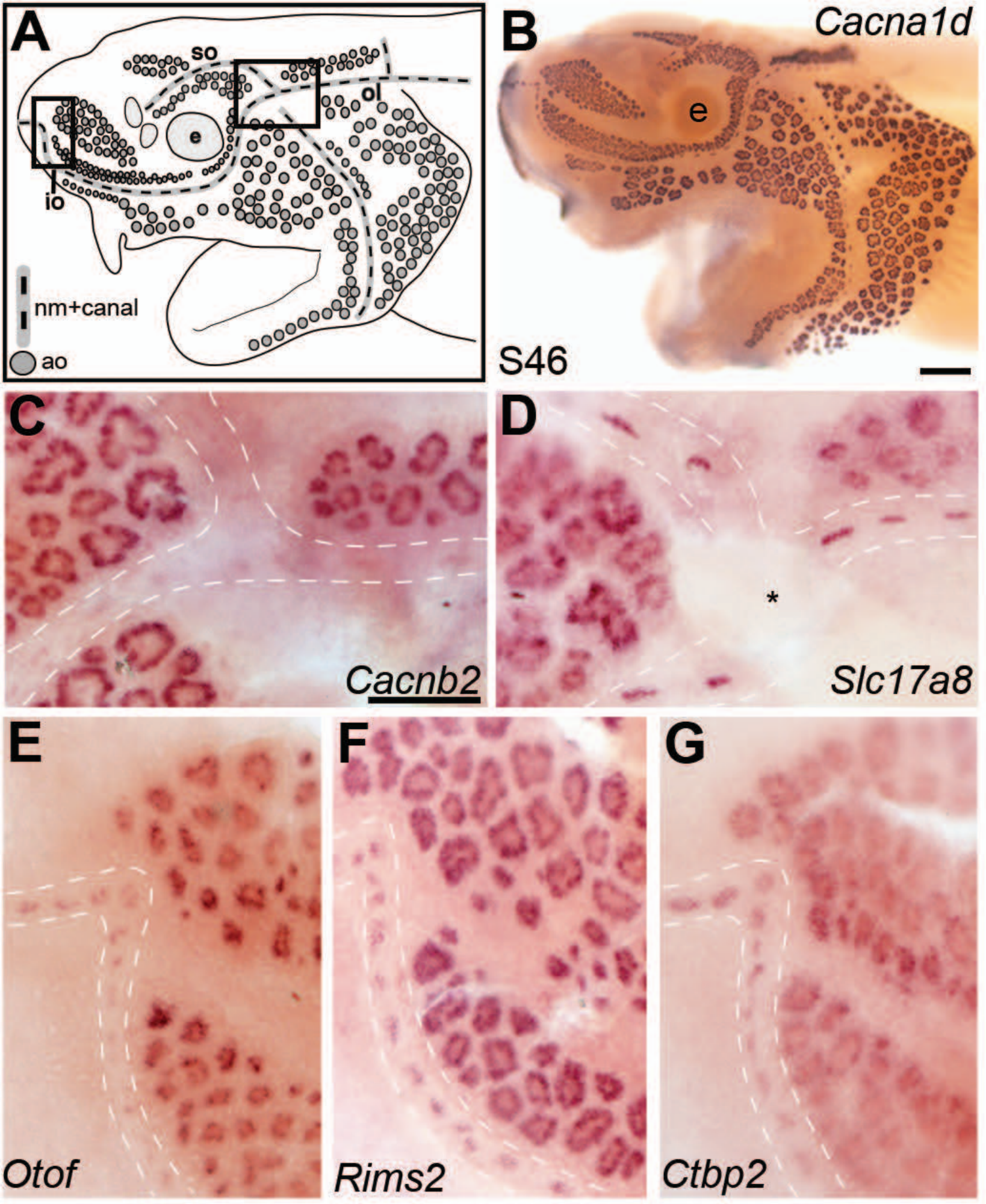
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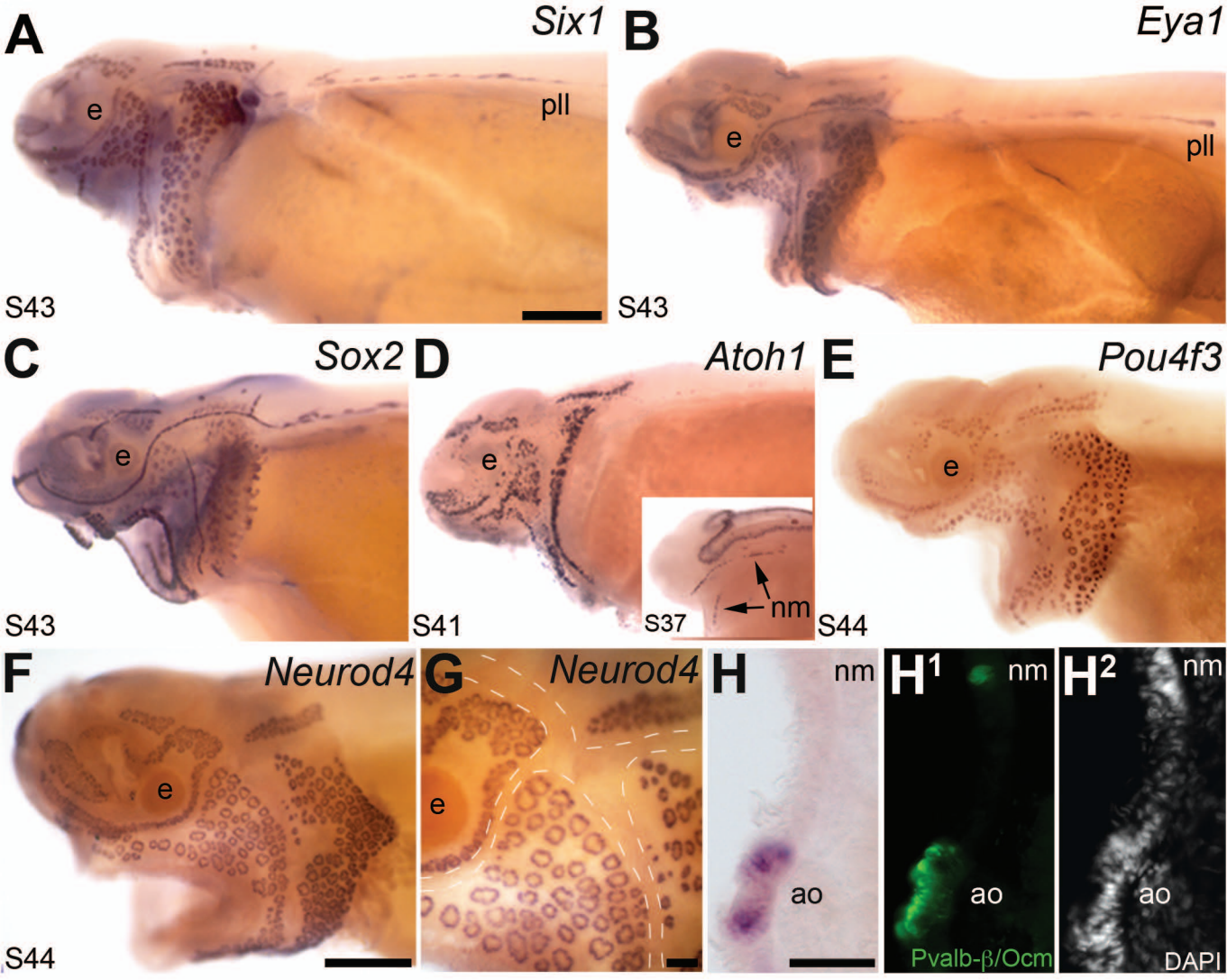
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